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Detection of DNA Sequence Polymorphisms in Human Genomic DNA by Using Denaturing Gradient Gel Blots

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Summary

Denaturing gradient gel electrophoresis can detect sequence differences outside restriction-enzyme recognition sites. DNA sequence polymorphisms can be detected as restriction-fragment melting polymorphisms (RFMPs) in genomic DNA by using blots made from denaturing gradient gels. In contrast to the use of Southern blots to find sequence differences, denaturing gradient gel blots can detect differences almost anywhere, not just at 4–6-bp restriction-enzyme recognition sites. Human genomic DNA was digested with one of several randomly selected 4-bp recognition-site restriction enzymes, electrophoresed in denaturing gradient gels, and transferred to nylon membranes. The blots were hybridized with radioactive probes prepared from the factor VIII, type II collagen, insulin receptor, β_2-adrenergic receptor, and 21-hydroxylase genes; in unrelated individuals, several RFMPs were found in fragments from every locus tested. No restriction map or sequence information was used to detect RFMPs. RFMPs can be used as genetic markers, because their alleles segregate in a Mendelian manner. Unlike most other methods for detecting DNA sequence polymorphisms, a genomic DNA blot made from one gel can be hybridized consecutively with many (30 or more) different probes.

Introduction

Much of the recent progress in human genetics has been the result of the application of molecular methods to identify and map single-base sequence differences in genomic DNA. RFLPs, used as genetic markers, have permitted the construction of genetic maps of entire human chromosomes (Drayna and White 1985; Donis-Keller et al. 1987; Bernatzky and Tanksley 1986). Mutations can also be detected as RFLPs, when they change restriction-enzyme recognition sites. However, as useful as RFLPs are, the testing of restriction sites alone permits the comparison of only a very small fraction of the total DNA sequence within any region analyzed.

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Many molecular methods have been devised to detect DNA sequence differences that cannot be detected as RFLPs. In denaturing gradient gel electrophoresis, sequence differences in otherwise identical fragments often cause them to partially denature at different positions in a gradient of denaturants (Fischer and Lerman 1983; Lerman et al. 1986). Even fragments differing by a single base pair often migrate differently in denaturing gradient gels. By comparing the melting behavior of sets of DNA fragments from different genotypes side-by-side in denaturing gradient gels, the fragments with sequence differences in the least stable (first) melting domain are identifiable because of their altered gel positions.

Denaturing gradient gel electrophoresis has been used to compare the melting behavior of cloned DNA fragments (Fischer and Lerman 1983), fragments amplified by PCR (Sheffield et al. 1989; Abrams et al. 1990; Traystman et al. 1990), and heteroduplexes of labeled, cloned fragments and their genomic counterparts (Myers et al. 1988). Denaturing gradient gels have previously been used to examine the melting be-

havior of genomic DNA fragments of *Drosophila* mutants directly (Gray et al. 1991). In these experiments, genomic fragments from the *rosy* locus were visualized by hybridization of radioactive probes to nylon hybridization blots prepared from denaturing gradient gels.

In the experiments described here, a denaturing gradient gel blot strategy similar to that of the *Drosophila* experiments was used to detect sequence polymorphisms in human genomic DNA. Because the complexity of the human genome (3.4×10^9 bp) is much larger than that of the *Drosophila* genome (1.8×10^8 bp; Lewin 1979), the autoradiographic exposure time necessary to visualize very short (250–600-bp) human genomic DNA fragments was expected to be too long to be of practical use. However, as shown in the examples below, satisfactory autoradiographs were obtained from blots of human genomic DNA prepared from denaturing gradient gels, after exposure times similar to those used for conventional Southern blots (2–12 d; Southern 1975). Examples of restriction-fragment melting polymorphisms (RFMPs) in the human genes encoding factor VIII, type II collagen, insulin receptor, β_2-adrenergic receptor, and 21-hydroxylase are shown.

Material and Methods

DNA Probes

Probe DNAs were prepared from clones supplied by other investigators. The factor VIII gene probe was supplied by Dr. Jane Gitschier (Wood et al. 1984). The type II collagen gene cosmid clone CosHcolI was a gift from Drs. Ellen Solomon and Julian Borrow (Cheah et al. 1985). The insulin receptor clone pTX contains bp 63–4443 of the human cDNA (Ullrich et al. 1985); this plasmid was a gift from Dr. Andrzej Krolewski. The human β_2-adrenergic receptor clone, pTF3, was supplied by Dr. Pablo Gejman (Kobilka et al. 1987*a*). The 21-hydroxylase gene clone pC21/3c was supplied by Dr. Perrin White (White et al. 1986).

Preparation of Genomic DNA Samples

Genomic DNA was prepared from peripheral blood leukocytes by using a modified standard method (Bell et al. 1981). Heparinized blood samples (10 ml) were mixed with 30–35 ml of cold (4°C) cell lysis buffer (0.32 M sucrose, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100) in 45-ml disposable plastic tubes. Nuclei were pelleted by centrifuging the tubes at 2,500 *g* for 15 min at 4°C. The dark-red supernatant was carefully removed, and the nuclear pellet was resuspended by vortexing in 5 ml of cold nuclear lysis buffer (75 mM NaCl, 24 mM EDTA pH 8.0). This suspension was then transferred to 13-ml polypropylene centrifuge tubes (Sarstedt 60.541). SDS was added to 1%, proteinase K was added to 100 μg/ml, and the suspension incubated at 55°C for 1–24 h. The lysate was extracted once with phenol/chloroform (1:1, equilibrated with 0.5 M Tris-HCl pH 8.0). The DNA was precipitated in isopropanol, and the pellet was washed once with 70% ethanol. The dried pellets were dissolved in TE (10 mM Tris-HCl, 1 mM EDTA pH 7.5) at ~1 μg/μl. Genomic DNA was digested in the supplier's buffer by using 1–2 units of restriction enzyme/μg DNA for 1–24 h. The volume of the digested DNA sample was kept under 15 μl, for convenience in loading gels. After digestion, 5 × sample loading buffer (80% glycerol, 10 mM Tris-HCl pH 8.0, 25 mM EDTA, bromphenol blue) was added to each DNA sample.

Preparation and Electrophoresis of Denaturing Gradient Gels

The denaturing gradient gel electrophoresis apparatus used was designed by Leonard Lerman (Fischer and Lerman 1979) and was obtained commercially (model LP-102; Green Mountain Lab Supply, Waltham, MA). Two or three gels were prepared and electrophoresed simultaneously according to a method described elsewhere (Gray et al. 1991). All gels were made by using glass plates 7 inches wide, 8 inches long, and 3/16 inches thick; polystyrene spacers 0.75 mm thick; and combs made from teflon. The combs had 20 wells, each 4 mm wide and 1.5 cm deep. The gradient maker was purchased from Hoeffer (model SG100). An immersible heater/circulator (PolyScience model 70) was used to keep the aquarium buffer at a uniform temperature of 60°C.

All gels had a wide range of denaturant concentration, such as 20%–80% (100% = 7 M urea, 40% formamide). To prepare gels, stocks of 0% and 100% denaturant/6.5% acrylamide (37.5:1 acrylamide:bisacrylamide) mixes in TAE buffer (40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA pH 7.4) less than 1 m old, were combined as needed for the intended denaturant concentration range, degassed 3 min under high vacuum, and placed on ice. Next, ammonium persulfate (1/200 vol of a 20% stock) and 1/2,000 vol of TEMED (N, N, N′, N′-tetramethylethylenediamine) were added to each of the two gel mixes. Gels were poured one at a time, over 5 min, in

succession. Gels were allowed to polymerize for at least 1 h before loading.

After polymerization, the upper buffer chamber was filled with TAE; the combs were removed, and the genomic DNA samples were loaded by using a pipettor. The apparatus and loaded gels were placed in the aquarium filled with preheated (60°C) TAE buffer, the DNA samples were electrophoresed overnight for 17–18 h at 65–85 V. After electrophoresis, the gels were stained in ethidium bromide (1 μg/ml) for 5 min, rinsed in water, and examined over a long-wave UV source.

Preparation of Nylon Blots

After the staining, the gels were soaked in TE for 5–10 min and then were transferred to a piece of blotting paper immediately before assembly into a "sandwich" in a tray filled with TE, as follows: First, a rigid plastic support was laid down, then a Scotchbrite pad, and then a piece of blotting paper. Next, the gel was laid down, with the paper side down. A presoaked (in TE) piece of nylon membrane (Hybond-N$^+$; Amersham) was placed on the gel by using forceps. Finally, two layers of blotting paper, another Scotchbrite pad, and the other rigid plastic support were placed on top of the sandwich. The stack was placed between the guides of the electrotransfer apparatus, previously filled with TE, with the nylon membrane between the anode (+) and the gel. DNA was electrotransferred from the gel to the blot for 2 h at the highest voltage possible (by using a Bio-Rad model 200/2.0 power supply), keeping the temperature of the buffer at 55°C or less (typically 30–80 V with a current of 0.6–2.0 A). After electrophoresis, the nylon blots were soaked in a tray of 0.5 M NaOH for 30 min, neutralized in 0.5 M Tris-HCl pH 8.0 for 5 min, and finally soaked in 6 × SSC (1 × = 0.15 M NaCl, 15 mM sodium citrate pH 7.4) for 5 min. The blots were air-dried for 5–10 min and then baked in a vacuum oven at 80°C for 1–2 h until the blots were absolutely dry.

Preparation of Probes

The DNA fragments used as probes were purified from their respective plasmid and phage vectors. The *Eco*RI fragments in the type II collagen gene recombinant cosmid CosHcolI were subcloned as separate recombinant pEMBL9 plasmids (Dente et al. 1983). Digested plasmid DNAs were electrophoresed in 4.5% acrylamide gels, for long enough to separate the inserts from the vector fragments (Maniatis et al. 1975). After electrophoresis, the gels were stained in ethidium bromide (1 μg/ml) for 10 min and were destained in water. Gel slices containing the DNA insert fragments were placed in small dialysis bags with 300 μl of 0.05 × TBE (1 × = 89 mM Tris-borate pH 8.3, 25 mM disodium EDTA), and were electrophoresed in a minigel apparatus in 0.05 × TBE at 500 V for 10 min. The DNA fragments were precipitated in 95% ethanol and were resuspended in small volumes of TE.

Purified DNA fragments were labeled by using the random primer extension method (Feinberg and Vogelstein 1983). Typically, about 1–200 ng of insert DNA was labeled with ^{32}P-dATP and ^{32}P-dCTP by using the conditions described by Hodgson and Fisk (1987), except that the incubation time was reduced to 30 min. The labeled DNAs were separated from unincorporated nucleotides by fractionation on 2 ml Sephadex G-50-150 (Sigma Chemicals) columns.

Hybridization of Blots with Radioactive Probes

Baked filters were hybridized with radioactive probes in the same way as Southern blots are done in our laboratory, by using the 7% SDS/sodium phosphate hybridization mix (Church and Gilbert 1983). Blots were placed in plastic bags (Micro-Seal; Dazey, Industrial Airport, KS), were rinsed with 6 × SSC, and were incubated with 25–50 ml of hybridization mix (1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, 7% SDS) for at least 1 h at 65°C. Probe DNA labeled with ^{32}-P (at least 2 × 10^7 dpm) was added to sonicated salmon sperm DNA (10 mg/ml) equal to 1% of that of the hybridization mix. The probe/salmon sperm DNA was heat-denatured for 3 min at 100°C, cooled on ice, and added to the hybridization bags. The blots were hybridized overnight (~16 h) at 65°C. The blots were washed after removal of the probe mix by incubation twice for 15 min each at 65°C in 500 ml of 2 × SSC, 0.5% SDS; then once for 30 min at 65°C in 500 ml of 1 × SSC, 0.5% SDS; and finally once for 30 min at 65°C in 500 ml of 0.1 × SSC, 0.5% SDS. The blots were air-dried for 15–30 min, covered with plastic wrap, and exposed to X-ray film (Kodak XAR-5) at −70°C in the presence of an intensifying screen for 1–10 d.

Removal of Probes

After an adequate autoradiographic exposure was obtained with the first probe, the nylon blots were usually rehybridized many times again (up to 50 times, for some blots) with new probes. Before the reprobing, blots were boiled in distilled water for ~1 min by using

a stainless steel tray placed over a large bunsen burner, to remove unwanted probe.

Results

Denaturing Gradient Gel Blot Strategy

Denaturing gradient gels can detect DNA sequence differences on the basis of physical properties of DNA fragments (Fischer and Lerman 1983). If the experimental objective is prenatal diagnosis or carrier testing for genetic disorders or is identification of new probes that detect polymorphisms useful as genetic markers, the best DNA markers are both easily detectable and highly informative. In such applications, it is not necessary that *every* sequence difference in each region analyzed be detected. The average density of sequence polymorphisms in most organisms has been extrapolated from the linear density of RFLPs; the density of human DNA sequence polymorphisms is thought to be ~0.3%, even lower (0.1%) on the X chromosome (Hofker et al. 1986).

In the following sections, examples of the denaturing gradient gel blot strategy for finding RFMPs in human genomic DNA are described and shown. In these human DNA experiments, the experimental strategy was that used previously by Gray et al. (1991) to compare predominantly first-melting domains. Optimal fragment lengths for this analysis were 250–700 bp, because these usually have at least two melting domains, each 100–200 bp in length. In the absence of any knowledge of either the melting behavior of any of the genomic fragments tested or where sequence differences were, five or six different 4-bp recognition-site restriction digests were compared. A small collection of inexpensive 4-bp restriction enzymes was used, including *Alu*I, *Dde*I, *Dpn*II, *Hae*III, *Hin*fI, *Rsa*I, and *Scr*FI. In previous experiments, analysis of several different digests increased the amount of sequence analyzed, because first-melting domains of some fragments overlapped higher-melting domains of other fragments. The DNA was electrophoresed long enough (16–21 h) for most (80%–90%) fragments to reach a gradient position where at least their first-melting domains would denature; wide-denaturant-range gels (e.g., 20%–80%) were used exclusively. After electrophoresis, DNA fragments were electrotransferred from the gels to nylon hybridization blots; blots were repeatedly hybridized with radioactive probes, as in Southern blot procedures.

Examples of RFMPs Found at Various Human Loci

Factor VIII gene.—Much RFLP data shows that the autosomes have a much higher density of sequence polymorphisms than does the X chromosome (Hofker et al. 1986). The factor VIII gene is a large (186 kb) gene that has been the subject of many searches for RFLPs (Gitschier et al. 1984, 1985). Only four factor VIII gene RFLPs have been found, in introns of the 3′ one-third of the gene (Antonarakis et al. 1987). Genomic DNA from normal individuals (not hemophilia A patients) was digested with *Hin*fI, electrophoresed in a 20%–80% denaturing gradient gel, and transferred to a blot. The blot was hybridized with a ^{32}P-labeled probe prepared from a fragment that includes exon 26, was washed, and was exposed to X-ray film. One of the three bands detected had polymorphic melting behavior, with two allelic forms (fig. 1, *top*). The bands higher on the blot (e.g., fig. 1, lanes 2 and 4) stopped where the denaturant concentration was 37%; the lower allele stopped at 37.5% denaturant, ~1.5 mm lower than the high form (e.g., lanes 3 and 5). The DNA electrophoresed in lane 5 was from a female heterozygous for both RFMP alleles. Most (77%) X chromosomes carried the high RFMP allele; the low allele was present in 23% of 196 X chromosomes tested (data not shown). In the original DNA sequencing experiments for the factor VIII gene, a base difference between a cDNA and a genomic clone was found in the 3′ untranslated region of exon 26 (Wood et al. 1984). At position 8728, there was a G in the cDNA sequence and an A in the genomic clone. Because both clones were obtained from different nonhemophiliac individuals, and because the base difference does not change the amino acid sequence, it was a silent polymorphism. The *Hin*fI RFMP may be caused by this same sequence polymorphism.

To determine the mode of inheritance of the factor VIII gene exon 26 *Hin*fI RFMP alleles, genomic DNA samples from individuals in four generations of a small family were tested. The DNA was digested with *Hin*fI, electrophoresed in a 20%–80% denaturing gradient gel, transferred to a blot, and hybridized with the exon 26 probe (fig. 1, *bottom*). No exceptions to Mendelian inheritance of either these RFMP alleles or any of the RFMP alleles discussed in the following sections were found either in this family or in any other families tested (Reindollar et al., in press; Krolewski et al., in press).

Type II collagen gene (COL2A1).—The type II collagen gene includes 54 exons distributed over 30 kb on chro-

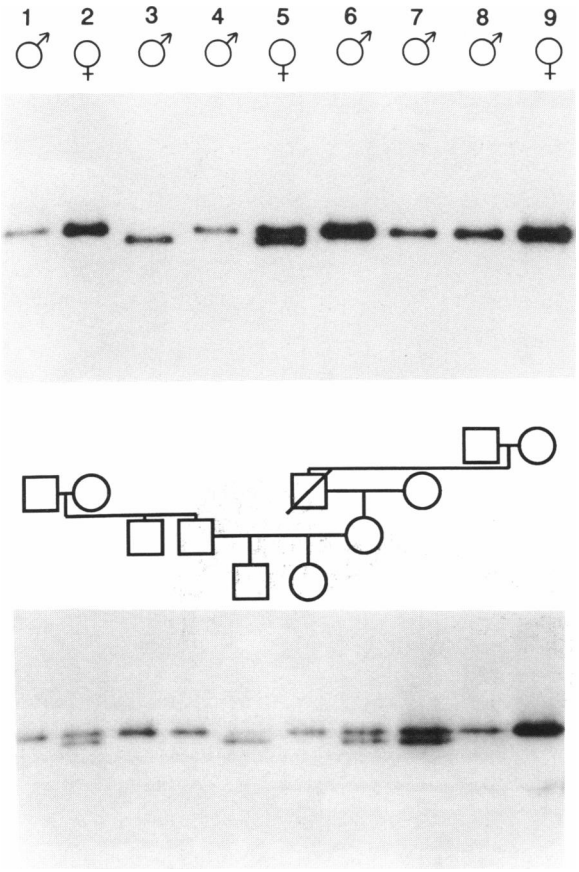

Figure 1 *Top,* RFMP in exon 26 of factor VIII gene. Genomic DNA (10 μg) from normal control volunteers was digested overnight with *Hin*fI, electrophoresed in a 20%–80% denaturing gradient gel at 82 V for 18.3 h, and transferred to a nylon blot. The blot was hybridized overnight with a probe prepared from a factor VIII cDNA subclone that includes all of exon 26 and was washed and then exposed to X-ray film for 4 d. The gender of each of the volunteer individuals is indicated at the top of each lane; all male individuals are hemizygous for factor VIII and almost all other X chromosome genes. *Bottom,* Mendelian inheritance of exon 26 RFMP. Genomic DNA from members of a small four-generation family (not known to carry mutations in the factor VIII gene) was digested with *Hin*fI, electrophoresed, and blotted as above; the blot was hybridized with the exon 26 probe and was washed and then exposed to X-ray film for 4 d. The pedigree is aligned with the lanes. Circles represent females, and squares denote males.

mosome 7 (Cheah et al. 1985; Sangiorgi et al. 1985; Ala-Kokko and Prockop 1990). Several RFLPs have been found in the gene (Sykes 1983; Strom 1988; Sykes et al. 1985), as have 12 additional sequence differences among three different sequenced alleles (Ala-Kokko and Prockop 1990).

A denaturing gradient gel blot of *Hae*III-digested genomic DNAs from unrelated healthy individuals was prepared and hybridized with a probe prepared from a 4.8-kb *Eco*RI fragment of the type II collagen cloned gene (Cheah et al. 1985). Six different fragments (designated A–F) were detected on the blot; fragments A, B, and D were polymorphic (fig. 2). Fragment A had two phenotypes; individuals represented by lanes 3, 4, 6, 12, 15, 17, and 18 of figure 2 were heterozygous for both common RFMP alleles. Fragment B had a diffuse and smeared phenotype, frequently found in previous *Drosophila* denaturing gradient gel blot experiments (Gray et al. 1991). Fragment B had at least two phenotypes; the higher form was visible in the DNA of individuals in lanes 1, 2, 6–8, 10–12, 14, 16–18. Because both alleles of this fragment had a diffuse appearance, it was not possible to determine the dosage of each on this blot; heterozygotes were not easily differentiated from those with two copies of the higher allele. Strategies that address this potential limitation for diffuse fragments are presented in a section below. Fragment D had three distinct phenotypes, with all individuals having at least one copy of the highest allele. Individuals in lanes 1, 6–8, 12, and 17 were heterozygous for the less common (6 of 36 chromosomes shown) middle allele; individuals in lanes 10 and 16 were heterozygous for the least common (only 2 of 36 chromosomes shown), lowest RFMP allele. Fragment C was not polymorphic, and the appearances of fragments E and F (somewhat diffuse, trailing upward at the edges) revealed that they had not been electrophoresed long enough to denature any of their melting domains. It was not possible, by using this blot (30%–80%; 82 V for 16.5 h), to determine whether fragments C, E, and F were polymorphic.

To see whether the 4.8-kb *Eco*RI probe RFMP result was typical of the entire type II collagen gene, probes were prepared from other subcloned portions of the gene (Cheah et al. 1985; Ramirez 1989). A 7.3-kb *Eco*RI fragment was used as a probe for *Hae*III genomic fragments (from normal volunteers) on a 20%–80% denaturing gradient gel blot; of the seven different *Hae*III fragments detected, five (A, C–E, and G) were polymorphic (fig. 3). The fragment E RFMP was difficult to see on this blot (compare lanes 5 and 6 of fig. 3); this RFMP might be more clearly observed after electrophoresis in a narrow-range denaturing gradient gel. Fragments B and F did not demonstrate polymorphism among the individuals tested. The

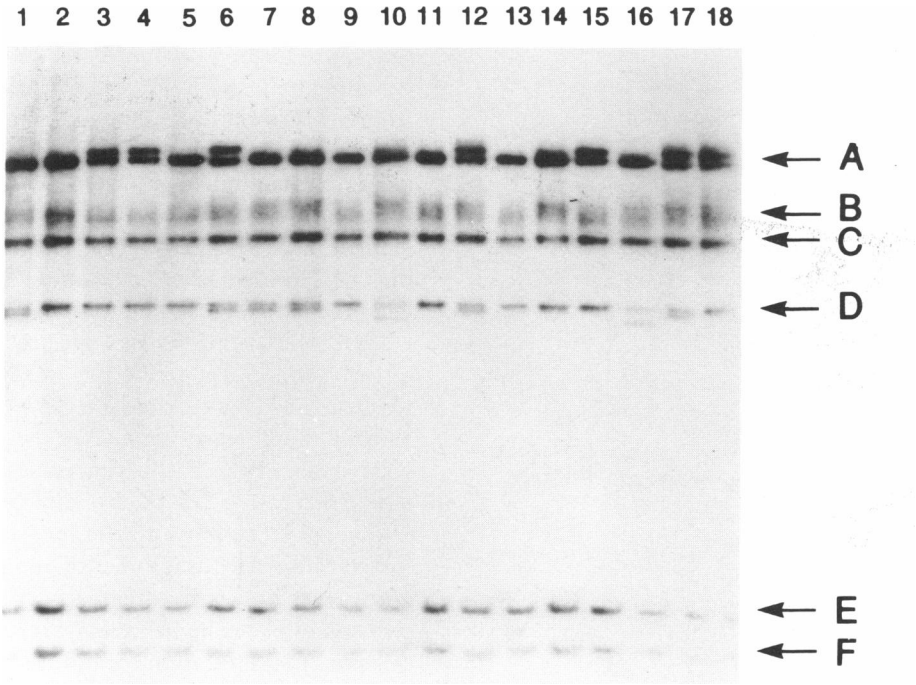

Figure 2 RFMPs in *Hae*III fragments of the type II collagen gene. Genomic DNA (10 μg) from unrelated volunteers was digested with *Hae*III and electrophoresed at 82 V for 16.5 h in a 30-80% denaturing gradient gel. After electrophoresis, the DNA was electrotransferred to a nylon blot and hybridized with a probe prepared from a 4.8-kb *Eco*RI fragment from the type II collagen gene. After the washes, the blot was exposed to X-ray film for 6 d. Six fragments (A–F) were detected and are discussed in the text.

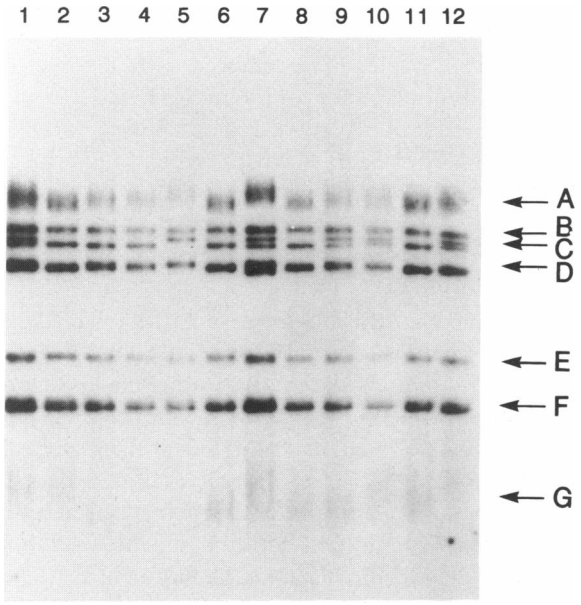

Figure 3 More *Hae*III fragment RFMPs in type II collagen gene. Genomic DNA from unrelated volunteers that was digested with *Hae*III was electrophoresed at 73 V for 21.3 h in a 20%–80% denaturing gradient gel. The blot prepared from this gel was hybridized with a probe prepared from the 7.3-kb *Eco*RI fragment from the cosmid CosHcol1 (Cheah et al. 1985). Blot exposure time was 6 d. The fragments A–G are discussed in the text.

RFMPs detected by the 7.3-kb *Eco*RI probe illustrated the range of phenotypes commonly seen. Fragment A had a diffuse appearance, with a high (lane 7) and low RFMP allele (lane 6). Fragment A in lane 9 was diffuse also but was centered at a new position midway between the high and low alleles; this intermediate phenotype may be either a third allele or simply the heterozygous combination of the high and low RFMP alleles. Fragment C had a sharp and well-focused RFMP phenotype, with two common alleles and a less common third one (the lower allele in lane 10). The three different fragment D RFMP alleles were found in lanes 6 (homozygous for the middle RFMP allele) and 7 (heterozygous for the high and low RFMP alleles). The phenotype of fragment G was a long (more than 1 cm) smear; polymorphic differences in the position of the smear were detected (compare lanes 2 and 3).

Hybridization of a denaturing gradient gel blot of *Alu*I-digested genomic DNA with the 7.3-kb *Eco*RI type II collagen gene probe used above for the *Hae*III blot revealed three more RFMPs (fig. 4). Fragments B, D, and G were polymorphic, with sharp band phenotype RFMP alleles; fragment D had three phenotypes: a high allele in 25% (9 of 36 shown), a middle allele in 42% (15 of 36), and a low allele in 33% (12 of 36).

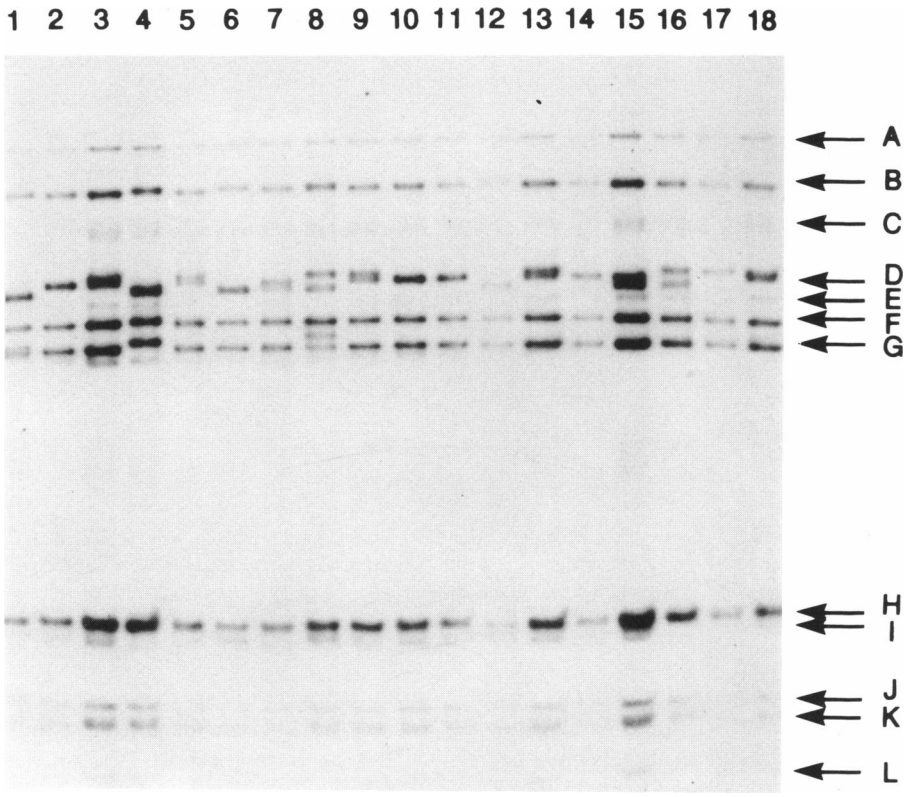

Figure 4 *Alu*I fragment RFMPs in type II collagen gene. Genomic DNA digested with *Alu*I was electrophoresed in a 20%–80% denaturing gradient gel at 80 V for 16.5 h and was transferred to a blot. The blot was hybridized with the same 7.3-kb *Eco*RI fragment probe used previously for hybridization with the *Hae*III blot (fig. 3). The fragments A–L are discussed in the text. Autoradiograph exposure time was 2 d.

Fragments H–L had not migrated far enough into the gradient for them to partially melt and stop in the gel; the ends of these bands trailed upward in the same manner as did fragments electrophoresed in nondenaturing gels. RFMPs in these *Alu*I fragments might be detectable by electrophoresis in a 50%–90% denaturing gradient gel.

A third type II collagen gene probe was prepared from the 9.3-kb *Eco*RI fragment (Cheah et al. 1985) and was hybridized to a blot prepared from a 20%–80% denaturing gradient gel of *Hin*fI digests of genomic DNA. Five fragments (A, B, D, E, and G) of the seven shown were polymorphic (fig. 5). Each of these RFMPs appeared to segregate independently of the others, even though they are closely linked physically; no RFMP haplotype patterns were discernible.

Genomic DNA samples were digested with additional restriction enzymes (*Rsa*I, *Dde*I, *Scr*FI, *Dpn*II, and others), electrophoresed in denaturing gradient gels, and transferred to nylon blots. These blots were hybridized with the type II collagen gene probes described above. As in the examples discussed above, the autoradiographs revealed RFMPs in more than half of all fragments detected (data not shown).

Insulin receptor gene (INSR).—Many human gene probes are available only as cDNA clones. On denaturing gradient blots, a cDNA probe will detect only exon-containing fragments, not fragments of entirely noncoding DNA from introns and flanking regions. The sequences of introns and DNA outside of genes are likely to be under less evolutionary pressure to be highly conserved and might be more polymorphic than are protein-coding exon sequences; most of the RFLP markers described are outside protein-coding DNA. Many of the exon-containing restriction fragments contain portions of introns and noncoding DNA, particularly if the exons are less than 500 bp in length. To further test the utility of using cDNA probes to detect sequence polymorphisms as RFMPs, probes from the insulin receptor gene cDNA were prepared.

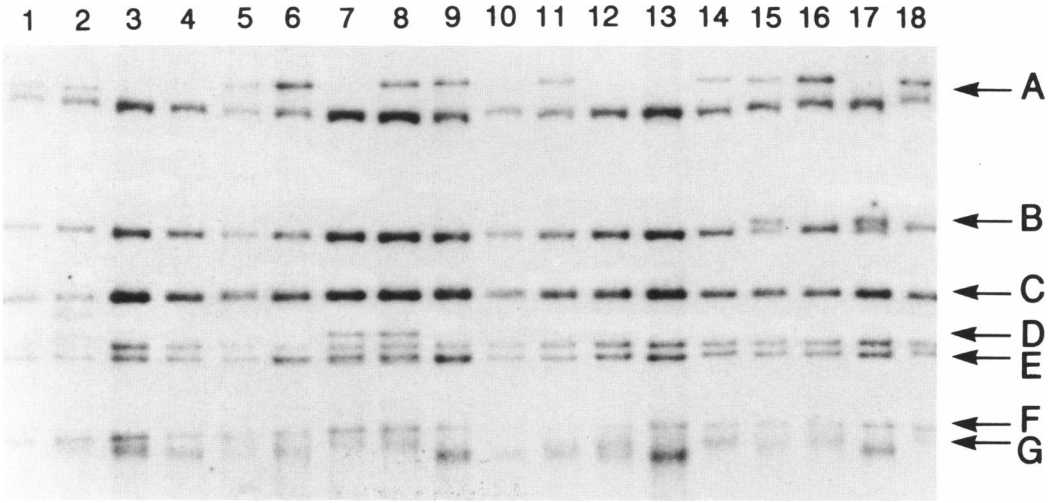

Figure 5 *Hin*fI fragment RFMPs in type II collagen gene. Genomic DNA digested with *Hin*fI was electrophoresed in a 20%–80% denaturing gradient gel at 82 V for 20.8 h and was transferred to a blot. The blot was hybridized with a probe made from the 9.3-kb *Eco*RI fragment from the type II collagen gene. The fragments A–G are discussed in the text. Autoradiograph exposure time was 11 d.

The insulin receptor gene is located on chromosome 19 and consists of 22 exons distributed over 130 kb of DNA (Seino et al. 1989). Several RFLPs have been found in the gene (Elbein et al. 1986; Cox et al. 1988).

A probe was made from the 5′ 1.1-kb *Eco*RI fragment of the cDNA; this fragment contains the first three exons, encoding part of the alpha peptide (Ullrich et al. 1985). This probe was hybridized with a 20%–80% denaturing gradient blot of *Alu*I digests of genomic DNA; four different fragments were detected on the blot. The upper two bands were polymorphic; the lowest bands, in the most denaturing region of the gradient, were diffuse in appearance and not polymorphic (fig. 6). There were at least four different RFMP alleles of the highest *Alu*I fragment; examples of all four can be observed in the DNA of the individuals in lanes 18–19 of figure 6. The frequency of each of the four alleles in the 40 chromosomes analyzed was as follows: allele A1, 5 chromosomes, or 12.5%; allele A2, 14 chromosomes, or 35%; allele A3, 18 chromosomes, or 45%; allele A4, 3 chromosomes, or 7.5%. Five different RFMP alleles of fragment B were found in the same group of 20 individuals (fig. 6). All had at least one copy of allele B5 (lane 1); half were heterozy-

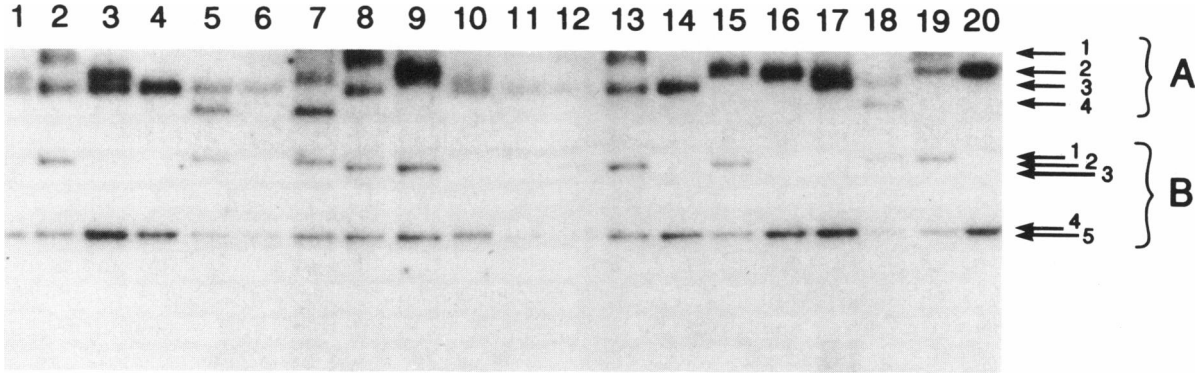

Figure 6 RFMPs in exon 1–3 region of insulin receptor gene. Normal volunteer genomic DNA samples were digested with *Alu*I, electrophoresed at 85 V for 17.25 h in a 20%–80% denaturing gradient gel, transferred to a blot, and hybridized a probe prepared from the 1.1-kb *Eco*RI fragment that includes the alpha-subunit portion (exons 1–3) of the insulin receptor cDNA (Ullrich et al. 1985). RFMP alleles of each fragment are indicated in the right margin by the arrows. Autoradiograph exposure time was 12 d.

gous for a second fragment B RFMP allele. Examples of each allele were present in DNA in lanes 5 (allele B1), 2 (allele B2), 8 (allele B3), and 10 (allele B4, ~0.5 mm above allele B5). Fourteen (70%) of the 20 subjects were heterozygous for at least one of these closely linked (within 45 kb) RFMPs. Denaturing gradient gel blots of DNA from other individuals revealed additional RFMP alleles of both *Alu*I fragments A and B (data not shown).

A second insulin receptor cDNA probe was made from the 3.3-kb *Eco*RI fragment that includes both the remaining coding sequence for the alpha subunit and the entire beta subunit (exons 4–22; Ullrich et al. 1985). This probe was hybridized with the same denaturing gradient gel blot of *Alu*I-digested genomic DNA previously hybridized with the probe made from the 7.3-kb *Eco*RI fragment of the type II collagen gene, shown in figure 4. Of the nine fragments shown in figure 7, five (B and D–G) were polymorphic. The RFMP alleles of fragments B and G demonstrated linkage disequilibrium in all subjects, except those in lanes 9, 14, and 17 of figure 7. Polymorphic fragments D and F had a diffuse phenotype; it was not possible to determine, on this blot, which individuals were heterozygous for alleles of these RFMPs. Electrophoresis of these *Alu*I fragments in a gel with denaturing gradient range of 30% or less might spread out the distances between RFMP alleles of fragments D and F. Hybridization with shorter probes would reduce the number of fragments detected and would eliminate the appearance of some comigrating bands. Polymorphic fragment E was difficult to score on this blot, because the RFMP alleles were very close together; electrophoresis in the narrow-range denaturing gradient gel might move the fragment E RFMP alleles farther apart.

β_2-Adrenergic receptor gene.—The B_2-adrenergic receptor gene is uncommon among human genes in having no introns; it has been suggested that this gene evolved from a related processed parent gene (Kobilka et al. 1987*a*; 1987*b*). Because it has no introns and is a single-copy gene, it seemed possible that its sequence would show little or no polymorphism. To test this, a β_2-adrenergic receptor gene probe was hybridized to the *Alu*I denaturing gradient gel blot previously hybridized with the collagen and insulin receptor probes, shown in figures 4 and 7, respectively. The probe was made from a 2-kb *Eco*RI cDNA fragment that included the entire coding region of the gene (Kobilka et al. 1987*a*). Both *Alu*I fragments detected on this blot were polymorphic (fig. 8). There were five different RFMP alleles of fragment A; examples of each were found in lane 1 (alleles A2 and A3), lane 5 (allele A4), lane 8 (alleles A4 and A5), and lane 13 (alleles A1 and A3) of figure 8. The frequencies of alleles A1–A5 were 2.7%, 30.5%, 33.3%, 30.5%, and 2.7%, respectively. Fragment B had two allelic

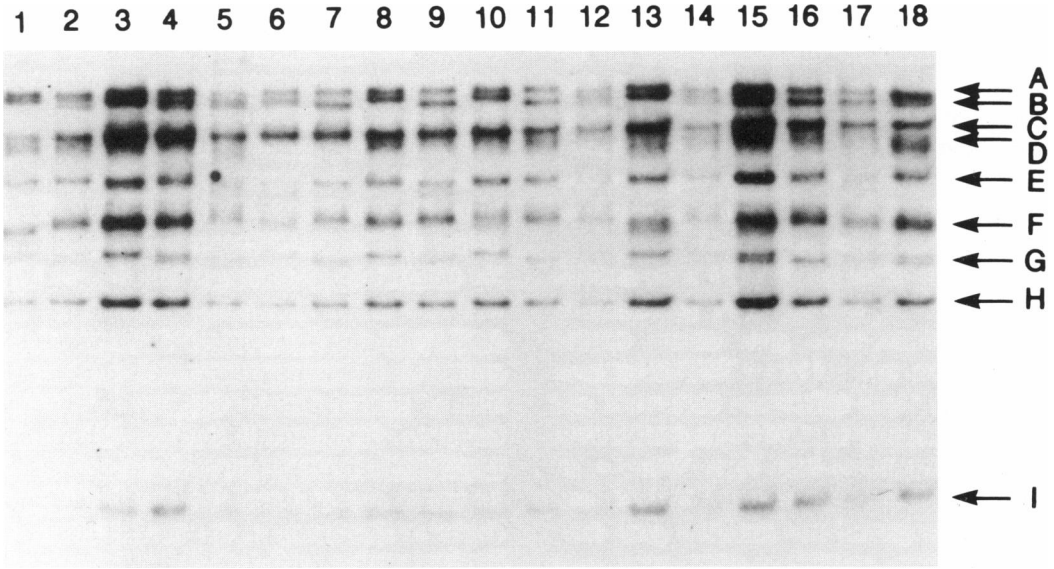

Figure 7 RFMPs in exon 4–22 region of insulin receptor gene. The same blot of *Alu*I-digested genomic DNA samples shown in fig. 4 was rehybridized with a probe prepared from the 3.3-kb *Eco*RI fragment of the full-length insulin receptor cDNA (Ullrich et al. 1985). Fragments A–I are discussed in the text. Autoradiograph exposure time was 9 d.

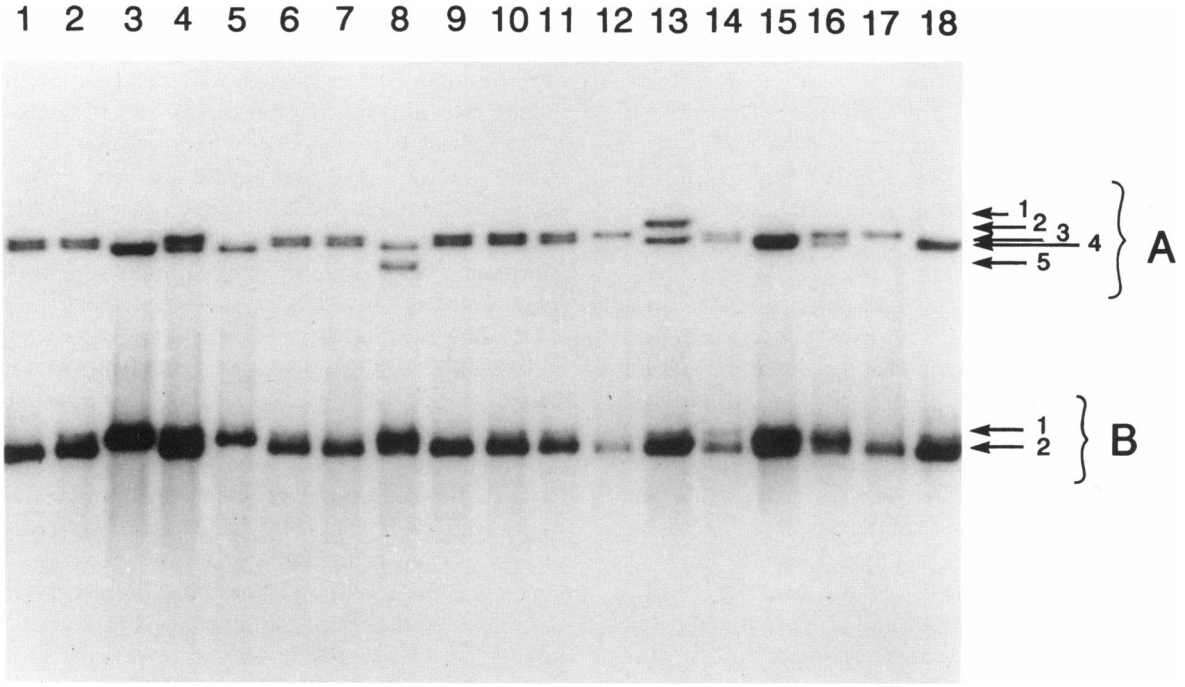

Figure 8 *Alu*I fragment RFMPs in β_2-adrenergic receptor gene. The *Alu*I denaturing gradient gel blot previously hybridized with probes from the type II collagen and insulin receptor genes (figs. 4 and 7) was hybridized with a probe made from the 2-kb *Eco*RI cDNA insert from the β_2-adrenergic receptor gene clone pTF; this clone includes the entire coding region of the gene (Kobilka et al. 1987*a*). Alleles of fragments A and B, indicated in the right margin, are discussed in the text. Autoradiograph exposure time was 5 d.

forms (B1 and B2); 22.2% of the 36 chromosomes tested had the high allele B1. All chromosomes with the A4 RFMP allele, except those in lane 18, also had the B1 allele. Perpendicular denaturing gradient gel electrophoresis of the cloned gene digested with *Alu*I demonstrated that fragment A was the 327-bp fragment from the 3′ half of the protein-coding sequence and that fragment B was the 838-bp fragment that contains the 5′ half of the protein-coding sequence (data not shown; Kobilka et al. 1987*a*).

21-Hydroxylase gene (CYP21).—*CYP21* is located on human chromosome 6, in the highly polymorphic major-histocompatibility-locus gene cluster (Miller and Morel 1989). The true *CYP21* (*CYP21B*) consists of 3,400 bp, with 67 base differences between it and the closely linked pseudogene (*CYP21A*); probes from either gene detect fragments from both (White et al. 1986). Gene conversions between *CYP21A* and *CYP21B* often cause partial and complete gene deletions and duplications, as well as conversions of a portion of one of the genes to a portion of the other. Although all of these events can cause mutations that eliminate 21-hydroxylase activity and cause congenital adrenal hyperplasia, a large proportion of normal individuals have been shown to have evidence of A/B gene conversions (White et al. 1987; Urabe et al. 1990). The increased genetic activity at this locus would suggest that it might be even more polymorphic than any of the genes tested above.

A denaturing gradient gel blot of *Alu*I-digested genomic DNA was hybridized with a probe prepared from the full-length *CYP21B* cDNA (White et al. 1986). All of the *Alu*I fragments were polymorphic on the denaturing gradient gel blot (fig. 9). Fragment A had three RFMP alleles (fig. 9, lanes 1–3). The middle set of fragments had many different patterns, probably caused by the comigration of the different RFMP alleles of several distinct fragments. The lowest *Alu*I fragment, designated B, had two RFMP alleles. The faint bands above fragment A in lanes 4, 6, 7, 10 and 14 varied also and may have been additional RFMP alleles of fragments in the middle cluster of bands. Taken together, each *Alu*I 21-hydroxylase gene RFMP pattern was unique in this random group of 14 individuals.

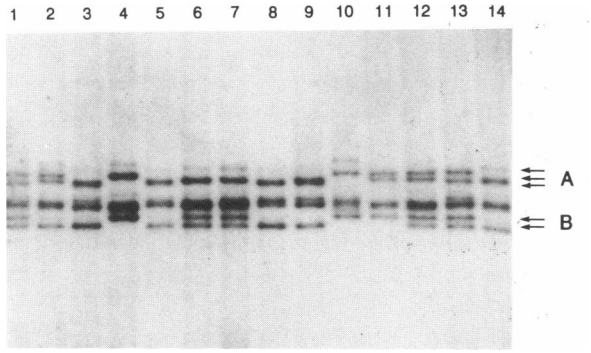

Figure 9 *Alu*I fragment RFMPs in 21-hydroxylase genes. *Alu*I-digested genomic DNA from unrelated individuals was electrophoresed at 81 V for 17.4 h in a 20%–80% denaturing gradient gel, transferred to a blot, and hybridized with a probe prepared from the full-length *CYP21B* cDNA (White et al. 1986). The arrows in the margin denote alleles of fragments A and B. Autoradiograph exposure time was 8 d.

Interpretation of Results

Some fragments did not form a sharp and well-focused band after denaturing gradient gel electrophoresis. When the electrophoresis was long (at least 1,500 V-h in a denaturing gradient gel 18 cm long), most fragments formed either a sharp band or a diffuse and smeared band. The diffuse smear patterns were usually the proper, reproducible melting phenotypes for some fragments; their appearance was most likely caused by the nearly simultaneous melting of more than one domain in the same fragment. In revealing the sequence differences, such fragments were as informative as sharply focused ones, as demonstrated in the previous *Drosophila* experiments (Gray et al. 1991). In the human DNA blots, the smear RFMP alleles were difficult to score in heterozygous individuals. We have not identified any set of gel or electrophoresis modifications, such as changes in the acrylamide or bisacrylamide concentrations, temperature, buffers, or denaturants, that converted the smear melting phenotype into a sharp-band phenotype. Digestion of genomic DNA with other restriction enzymes often rearranged the domain boundaries of the fragments under study and frequently redistributed, among two or more simple two-domain fragments that focused sharply, the DNA sequence in fragments that produced smeared phenotypes (Gray et al. 1991; M. Gray, A. Charpentier, K. Fusaris, and J. Myers, unpublished experiments).

As mentioned above, some fragments formed neither sharp, focused bands nor smears but instead appeared as slightly diffuse bands that trailed upward at the sides. In our previous experiments, such fragments were usually short (less than 250 bp), GC rich, and found in the most denaturing region of the gradient (Gray et al. 1991); such examples in the present study include fragments E and F in figure 2 and fragment H in figure 4. When electrophoresed in a more denaturing gel, such as a 50%–90% denaturing gradient gel, most of these fragments eventually focused; those that did not ran off the bottom of the gel either as completely double-stranded, or as completely denatured, without forming a partially melted structure (data not shown). In almost all of the examples of denaturing gradient gel blots shown above, a 20%–80% denaturant range was used; in each case, RFMPs were found. It is likely that, for each probe and restriction digest, a very different (from the one used) optimized set of electrophoresis conditions would reveal RFMPs more distinctly and might even reveal additional RFMP fragments.

Some RFMP differences were very small (0.5 mm or less) and difficult to score, except on the best of blots. These were retested by repeating their electrophoresis in denaturing gradient gels with a much narrower range of denaturant concentration. For example, one set of RFMP alleles was present at a position 60% of the distance from the top of a 20%–80% gel; its denaturant concentration for melting of the first domain was 56% (0.6 × 60% + 20%). The electrophoresis of this DNA was repeated in a denaturing gradient gel with a range of 41%–71% (15% on either side of that desired); the shorter gradient exaggerated the subtle RFMP differences.

Occasionally, more bands were present on a blot than could be accounted for by the probe used; these extra bands were usually the result of incomplete restriction-enzyme digestion of the genomic DNA. The partial digest bands were nearly always higher on the gel than were the fully digested fragments, and, because of the variable extent of partial digestion, their signal intensities always varied among different samples. Incomplete digestion was confirmed by the detection of additional bands after the blot was rehybridized with other probes.

When the sequence of the analyzed region was known, the number of fragments likely to be found on the blots was known; occasionally, one or two of the expected fragments were absent. A time-course genomic blot was useful for analyzing such discrepancies, as well as for determining the optimal denaturing gradient gel electrophoresis conditions for the fragments

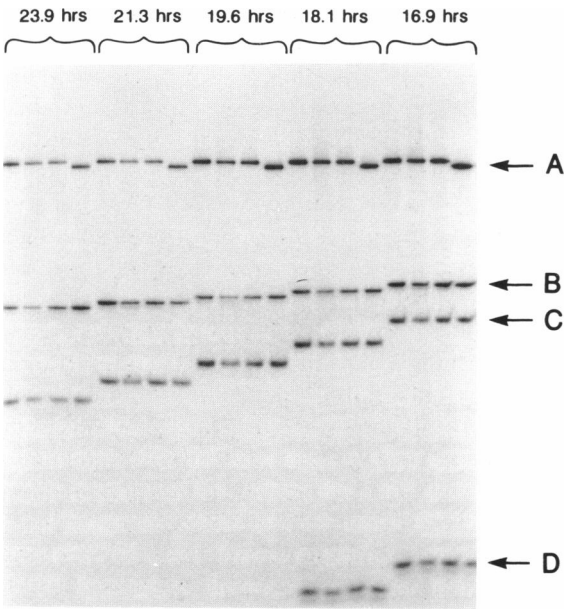

Figure 10 Time-course denaturing gradient gel blot. Genomic DNA samples (10 μg) were digested with *Hin*fI and electrophoresed at 85 V for different times, as shown (1,345–1,955 V-h), in a 20%–80% denaturing gradient gel. The blot was hybridized with a probe prepared from the factor VIII gene cDNA subclone that includes all of exon 26 and was washed and then exposed to X-ray film for 11 d. The entire gel is shown. The four fragments discussed in the text are designated A–D; fragment A is the same as that shown in fig. 1.

under study. In these experiments, the same set of digested DNA samples were electrophoresed on the same gel for different times (fig. 10). Four different genomic DNA samples were digested with *Hin*fI and were electrophoresed at 85 V for different times (16.9–23.9 h). Four fragments were detected on the blot by using the same factor VIII gene exon 26 probe used previously (fig. 1). Fragments A and B remained well focused, when the electrophoresis time was 18.1 h or more (fig. 10). Fragment A did not migrate farther into the gradient, even after an additional 7 h of electrophoresis; the positions of each fragment A RFMP allele remained unchanged. Fragment B formed bands that trailed up at the ends of the bands after 16.9 h but not after 18.1 h. Fragments C and D also formed bands that trailed up; they did not stop and form a sharp band, even after 23.9 h (in the case of fragment C). The rate of migration of these bands was dependent on their electrophoresis times and molecular weights. This blot revealed that the optimal denaturant concentration range for analysis of fragments A and B was low, such as 20%–60%. The time-course blot suggested that, if fragment C is to stop in the gradient, it should be electrophoresed even longer than 23.9 h and possibly in a more denaturing gel. Fragment D may be short and GC rich, as discussed above, because it never melted and stopped, even at 80% denaturants. Such time-course blots need to be made only once for each restriction enzyme used, because the same blots can be hybridized with many different probes.

Identification of RFMP Fragments

Although denaturing gradient gel blots can be used to detect sequence differences among fragments from the region detected by the probe, there is usually no information about the exact molecular-map location of an RFMP on the initial autoradiograph. The mobility of a DNA fragment in a denaturing gradient gel is dependent on sequence composition, not on length. It is often important to map exact locations of RFMP fragments after they are detected on denaturing gradient gel blots. If the DNA sequence of any such fragment is needed, precise mapping is helpful in minimizing the amount of sequence that must be obtained to find the base difference causing the RFMP. It was not difficult to determine an RFMP fragment's identity; any RFMP fragment can be mapped precisely.

Some genomic RFMP fragments have been mapped by subdividing the original fragment used as the probe and by then separately labeling them for rehybridization either to the denaturing gradient gel blot originally used to detect the RFMP or to similar blots. Additional information useful for mapping an RFMP fragment was the occurrence of previously mapped landmark restriction sites in small fragments. The identity of some bands on genomic blots has been determined using perpendicular denaturing gels (Fischer and Lerman 1983). In these gels, the direction of electrophoresis is perpendicular to that of the denaturing gradient; fragments loaded in a well spanning the entire width of the gel enter the gradient at all denaturant concentrations simultaneously and often form distinctive S-shaped curves. When the complete DNA sequence was known for the region detected by the probe, it was possible to construct a 4-bp restriction-site map. On the left, low-denaturant-concentration side of the gel, fragments migrated in a molecular-weight-dependent manner. Where the denaturant concentrations were higher, fragments lost mobility in an order dependent on the thermal stabilities of their first-melting domains. By following the curves from

left to right, the molecular weight of each fragment was correlated with its melting order. The left-to-right melting order in a perpendicular denaturing gradient was usually the same as the top-to-bottom melting order in "parallel" denaturing gradient gels (i.e., gels in which the direction of the electrical field was parallel to that of the gradient).

Discussion

Hybridization of denaturing gradient gel blots is a far more sensitive method for detecting DNA sequence differences than are traditional RFLP approaches. No sequence or restriction-map information for the region to be analyzed is necessary to detect RFMPs. Sequence differences do not have to be at restriction-enzyme recognition sites in order to be detected. Only a small proportion of the sequence in any region is included in restriction sites; only a small fraction of the known restriction enzymes are available to most investigators, often at high cost. In denaturing gradient gel electrophoresis, a sequence difference must be in the first-melting domain of a fragment to be detected easily. Much of the sequence in any region can be placed into such domains of small fragments by using restriction enzymes that cut frequently, producing fragments 250–700 bp in length. By using several different restriction enzymes, much of the sequence outside the first-melting domain of one fragment is redistributed to first-melting domains of other fragments. The choice of restriction enzymes used is not important, as long as many of the resulting fragments are in the correct size range. The same small set of restriction enzymes can be used to analyze many different genes. The 4-bp recognition-site enzymes used in these experiments are available from many suppliers and are among the least expensive restriction enzymes. RFMP alleles can be used as genetic markers; all of the RFMPs used in the present study demonstrate the Mendelian mode of inheritance (Krolewski et al., in press; Reindollar et al., in press; M. Gray, A. Charpentier, and K. Fusaris, unpublished experiments); a more detailed presentation of our experiments showing segregation of RFMP alleles will be presented elsewhere.

With denaturing gradient gel blots of genomic DNA, hundreds of individuals can be compared at many different loci, in a short period of time. The only requirements are (1) genomic DNA, (2) probe DNAs for the region of interest, and (3) small vertical acrylamide-gel apparatus capable of electrophoresis at 60°C. The skills required to prepare denaturing gradient gel blots are similar to those required to prepare Southern blots. With the procedure described above, denaturing gradient gel blots can be prepared in 1.5 d, or 1 d less than the time required for traditional agarose-gel Southern blots. A denaturing gradient gel can be cast in 2 h; after overnight electrophoresis, the blots can be prepared in 5 h. The blot from each gel can be used numerous times; some blots have been hybridized more than 50 times during the past 3 years, with little or no loss of signal.

Any type of single-copy sequence probe can be used to detect RFMPs on denaturing gradient gel blots; the method of labeling either DNA or RNA is not important. The sequence used as the probe can be from either a genomic clone (figs. 2–5) or a cDNA clone (figs. 1 and 6–10). The size of the probe determines how many fragments are visualized on each blot; the numbers of fragments detected on the blots above are representative of the results of all of our experiments. Probes made from cDNA fragments can often detect many fragments that include the sequences from many short exons. The signal intensity of bands detected by using cDNA probes varies according to the length of overlap between the probe and the genomic fragment that includes part of an exon; often, this length is very short (such as 50 bp), and the signal intensity of some bands is very low. For this reason, cDNA probes may not be the best probes to use for detecting mutations in genes with many small exons. Genomic fragments are probably better as probes for such genes, as long as the introns do not contain repeated sequences, because the signal intensity of each band does not depend on the length of the exons. As in traditional Southern blots, it must be established that any probe hybridized with a denaturing gradient gel blot detects fragments at one location in the genome.

There are many other methods for detecting single-base differences in genomic DNA; most of these were designed to detect and locate mutations in well-characterized genes. In one method, GC-rich DNA clamps are attached to genomic fragments during PCR amplification; mutations can be detected by denaturing gradient gel electrophoresis, even in second- and third-melting domains of these amplified fragments (Myers et al. 1988). In another approach, heteroduplexes of cloned, ^{32}P-labeled wild-type and genomic DNA strands are compared by denaturing gradient gel electrophoresis (Myers et al. 1988). This method nearly guarantees detection of fragments with base differences, because of the destabilizing effect of mismatches in the two hybrid fragments formed. Other

heteroduplex mismatch methods have been devised that use either RNAse A digestion at mismatches in RNA:DNA duplexes (Myers et al. 1988) or chemical cleavage at mismatches in DNA duplexes (Cotton et al. 1988). Sequence differences can even be detected in small single-stranded DNA fragments on neutral acrylamide gels (Orita et al. 1989). Most of these methods are extremely effective for detecting mutations in previously sequenced genes.

Another type of sequence polymorphism is that of short tandem repeat sequences with a variable number of repeat units. VNTR markers can be detected by using traditional agarose-gel Southern blots (Nakamura et al. 1987). Variation in the number of repeat units of dinucleotides is another source of polymorphic markers; such polymorphisms have been detected by electrophoresis of repeat-containing fragments amplified from genomic DNA by PCR (Weber and May 1989). These methods are restricted by the locations of the repeated sequences; some genes may not have nearby clusters of repeating dinucleotides, or VNTRs. Also, the occurrence of such repeats may be rare in some regions of the genome.

For rapid detection and mapping of new polymorphisms in uncharacterized DNA, these other methods for detecting sequence differences are less practical than are denaturing gradient gel blots. In most of these methods, information can be obtained from each gel electrophoresis experiment only once. In contrast, one denaturing gradient gel genomic DNA blot can be hybridized with many probes, providing 30 or more sets of results from each gel. There is some loss of signal after many hybridizations of the same blot; on average, there is a 50% reduction in signal intensity after 30 reprobings. Only conventional quantities of radionucleotides are used, and there is no requirement for expensive enzymes, oligonucleotides, and equipment such as programmed temperature cyclers, DNA sequencers, or oligonucleotide synthesizers. Little or no experimental optimization is required for every site analyzed.

The distribution of RFMPs found in the examples described above is consistent with the prediction that the average density of polymorphisms is 1 in 300 bp on autosomes (Hofker et al. 1986). Typically, in experiments with completely sequenced loci, about half of the sequence was included within 250–600-bp fragments after digestion with a typical 4-bp recognition-site restriction enzyme (Gray et al. 1991; M. Gray, unpublished experiments). Examination of denaturing gradient gel blots shows, on average, that about one-third of the sequence of the fragments detected is within first-melting domains. In general, about half of the fragments detected in the present study demonstrate RFMPs. Thus, in a typical 5-kb region, where one-third of the sequence in half of the region is compared by denaturing gradient gel electrophoresis, two or three RFMPs were usually detected, i.e., two or three polymorphisms in $1/3 \times 1/2 \times 5{,}000$ bp, or two or three in 833 bp—roughly 1 in 300—consistent with the earlier estimate based on RFLP density. So far, RFMPs have been found in every region examined, including the X and Y chromosomes (M. Gray, unpublished experiments); there have not yet been found any human probes longer than 4-kb that truly *do not* detect RFMPs.

It is not yet known what proportion of single base differences can be detected by using denaturing gradient gel blots, as described above. An earlier study detected and mapped 100 of 130 *rosy* mutations in *Drosophila melanogaster* by using denaturing gradient gel blots (Gray et al. 1991). Most of these mutations were found in GC-rich regions of the sequence. Thus, base differences in regions difficult to melt were not necessarily difficult to detect. Some sequence differences are probably not ever detectable by comparing the melting behavior of first-melting domains. Undoubtedly, many short regions of sequence will always be in higher-melting domains of all restriction fragments that include them. As discussed elsewhere, conservative transversions are difficult to detect because of their subtle affects on melting behavior (Myers et al. 1985; Gray et al. 1991). This should not be regarded as a barrier to finding new polymorphisms; the RFMP frequency suggests that only a very small proportion of sequence polymorphisms are likely to be conservative transversions.

With a more comprehensive detection of closely spaced sequence polymorphisms, it is possible to examine linkage disequilibrium much more carefully. Closely spaced RFMP alleles that appear to be in nearly perfect linkage disequilibrium (fig. 7, fragments B and G) have been found, as have pairs of RFMPs that seem to be completely discordant (fig. 5, fragments A, B, D, E, and G). With many polymorphisms, some exhibiting more than two phenotypes, all alleles can be followed in pedigrees by using RFMPs as codominant markers; haplotypes of groups of polymorphisms are easily assigned in studying families.

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